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	Examiner Name	C. Myers
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Title of the Invention: DETECTION OF ANTIBIOTIC RESISTANCES IN MICROORGANISMS		

SUBMISSION OF CERTIFIED TRANSLATION

Commissioner for Patents
Washington, D.C. 20231

April 3, 2003

Dear Sir:

Further to our Response filed February 6, 2003, attached are copies of the certified translation of priority documents DE199 16 610.2 and DE198 23 098.2

RESPECTFULLY SUBMITTED,					
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I, Susan ANTHONY BA, ACIS,

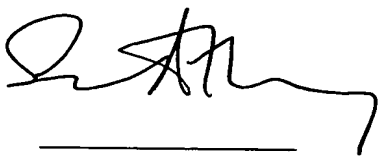
Director of RWS Group plc, of Europa House, Marsham Way, Gerrards Cross, Buckinghamshire, England declare;

1. That I am a citizen of the United Kingdom of Great Britain and Northern Ireland.

2. That the translator responsible for the attached translation is well acquainted with the German and English languages.

3. That the attached is, to the best of RWS Group plc knowledge and belief, a true translation into the English language of the accompanying copy of the specification filed with the application for a patent in Germany on 22 May 1998 under the number 198 23 098.2 and the official certificate attached hereto.

4. That I believe that all statements made herein of my own knowledge are true and that all statements made on information and belief are true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application in the United States of America or any patent issuing thereon.



For and on behalf of RWS Group plc

The 28th day of January 2003

FEDERAL REPUBLIC OF GERMANY

Certificate

Creatogen Biosciences GmbH

of

Augsburg/Germany

have filed a Patent Application under the title:

"Detection of antibiotic resistances in microorganisms"

on 22 May 1998 at the German Patent and Trademark Office.

The attached documents are a correct and accurate reproduction of the original submission for this Patent Application.

The German Patent and Trademark Office has for the time being given the Application the symbols C 07 H and C 12 Q of the International Patent Classification.

Munich, 24 June 1999

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Detection of antibiotic resistances in microorganisms

Detection of antibiotic resistances in microorganisms

Description

The invention relates to a process for detecting antibiotic resistance in microorganisms, in particular in bacteria, and to reagent kits which are suitable for implementing the process.

Zuckerkandel and Pauling (1965) who, in the article: "Molecules as documents of evolutionary history" were the first to point out the possibility of revealing the evolution of organisms by comparing the sequences of their appurtenant macromolecules, laid the foundation stone for developing an rRNA-directed in-situ hybridization for detecting pathogenic organisms. It was then Carl Woese who used this concept for working out the first natural classification system in prokaryotes (Woese, 1987). A further result of these investigations was that rRNA sequences exhibit so-called signature sequences which are typical of particular domains, phyla, families, genera and even individual species. Detecting these signature sequences with the aid of PCR primers or hybridization probes therefore makes it possible to identify bacteria on different taxonomic levels. In addition, the high number of rRNA molecules which are naturally present in the bacterial cell (10^4-10^5 in rapidly growing bacteria) increases the sensitivity of the method and made it possible to use in-situ hybridization techniques which employ rRNA as the target molecule. Using radioactively labeled oligonucleotide probes, Giovannoni et al. 1988 were the first to be able to detect rRNA in whole bacterial cells and, one year later, DeLong et al. (1989) carried out such an in-situ hybridization using fluorescence-labeled oligonucleotides.

Recently, this technique has been employed frequently, particularly in environmental microbiology. The

location of particular physiological groups (Wagner et al., 1993; Ramsing et al., 1993) and the influence of particular agents on the composition of the population of an ecosystem (Wagner et al., 1995) were the focus of interest in this connection.

However, this technique has also been employed successfully for detecting bacteria in the food hygiene sphere (Beimfohr et al., 1993). Medical microbiology is another field of microbiology in which rRNA-directed whole-cell hybridization is applied.

Thus, *H. influenzae* has been detected in throat swabs taken from children (Forsgren et al., 1994), *Candida* species have been detected in blood cultures and tissue samples taken from artificially infected animals (Lischewski et al., 1996, Lischewski et al., 1997), pathogenic *Yersinia* species have been detected in tissue sections, stools and throat swab samples (Trebesius et al., J. Clin. Microbiol. submitted), and salmonella have been hybridized as successfully in swabs (Nordentoftet al., 1997) as have bifidobacteria in stool samples (Langendijk et al., 1995).

As various investigations have demonstrated, the number of ribosomes in rapidly growing, heterotrophic bacteria depends heavily on the growth rate and the physiological activity of the organism (Schaechter et al., 1958). Since the quantity of bound probe is proportional to the quantity of rRNA, the state of growth of a cell can also be determined indirectly by way of the hybridization-mediated fluorescence (DeLong et al., 1989).

A comparison of the translation apparatus in eukaryotic cells and in bacterial cells shows considerable differences in the function and structure of the individual components. These differences create a therapeutic window for a series of active compounds

which intervene specifically in the bacterial translation process but which do not intervene in the eukaryotic translation process. Table 1 lists frequently employed antibiotics which intervene in the translation process in the bacterial cell. These active compounds possess the second highest worldwide market share, coming after the antibiotics which are directed against peptidoglycans.

However, the massive therapeutic employment of these substances leads to the emergence of resistances in clinical isolates and consequently to therapy failures. A number of causes may be responsible for the appearance of such a mutation:

(1) Change in the target site for the antibiotic
(2) Modification of the antibiotic
(3) Change in the transport of the antibiotic

In the case of the MLS antibiotics (macrolide, lincosamide, streptogramin B), which achieve their effect by blockading the ribosomal peptidyltransferase center, investigations carried out on clinical isolates lead to the conclusion that changes in the target site for the antibiotic are responsible for the development of resistance in the overwhelming majority of cases (Versalovic et al., 1997). Several variants are also conceivable in this connection.

(1) Mutation of ribosomal proteins
(2) Mutation of the rRNA
(3) Posttranscriptional modification

Whereas it was generally assumed previously that change in ribosomal proteins was mainly responsible for the development of resistance, experimental data of more recent origin militate against such a theory and instead support the thesis that changes which take place directly on the ribosomal RNA

(posttranscriptional methylation or mutation) lead to the development of resistance.

Table 1:

Class/Active compound	Use
Aminoglycoside/aminocyclitol antibiotics (Dihyro)streptomycin neomycin, paromomycin kanamycin gentamicin, tobramycin, amikacin, netilmicin, sisomicin spectinomycin	**Market share: 3%** Tuberculosis therapy; resistance frequent oral and topical use parenteral administration; resistance frequent new; broad spectrum (not Streptococci or Enterococci; ototoxic and nephrotoxic; blood level control) penicillinase-resistant gonococci
Lincosamides Lincomycin, clindamycin	Gram-positive bacteria and Gram-negative anaerobes; good penetration into bone tissue; in the case of toxin formers
Macrolides Erythromycin, roxithromycin, clarithromycin, Azithromycin	**Market share: 11%** Against Gram-positive and Gram-negative cocci, chlamydias and mycoplasmas, Helicobacter; good ability to traverse the membrane → intracellular bacteria
Tetracyclines Tetracycline, oxytetracycline, rolitetracycline, doxycycline, minocycline	**Market share: 3.5%** Broad spectrum, including chlamydias and rickettsias; predominantly bacteriostatic; resistance frequent; deposition on teeth in infants;

Thus, carbomycin was able to inhibit the in-vitro peptidyltransferase activity which was exhibited by protein-free 23S rRNA extracts (Noller et al., 1992). Furthermore, the affinity constants for the binding between erythromycin and ribosomal proteins, such as L15, which are claimed to be potential candidates for the appearance of resistance, are several orders of size less than those which were ascertained for complete ribosomes (Weisblum, 1995). In particular,

however, the lack of erythromycin-resistant clinical isolates which have a mutation in their ribosomal proteins indicates that this mechanism of resistance is more likely to be of little importance (Weisblum, 1995).

However, the other two mechanisms of resistance are frequently encountered, with a striking observation being that the target region for both changes relate [sic] to particular bases in domain V of the 23S rRNA (Brimacombe, 1990).

An adenine residue in *E.coli* position 2058 (numbering according to Brosius et al., 1981), which residue is located in the tertiary structure of the 23S rRNA between helices 73 and 74 (numbering according to Brimacombe et al., 1980), is the substrate for the modification by methylases of the erm family which have been isolated from various macrolide-resistant clinical isolates (Weisblum, 1995). An A → G transversion in this position also leads to a resistance phenotype in a number of phylogenetically dissimilar bacteria (*Mycobacterium intracellulare* (Meier et al., 1994), *H. pylori* (Versalovic et al., 1997) and *E.coli* (Sigmund et al., 1988, Vester and Garrett, 1987). Table 2 lists a number of other mutations in the peptidyltransferase center of the 23S rRNA which are found in resistant bacteria. The conserved nature of the positions which lead to MLS resistance, and their discovery in a very wide variety of phylogenetic groups (not only bacteria), demonstrates the general nature of this phenomenon. Since the discovery of these mutations is relatively recent, it is to be expected that modifications/mutations of the rRNA/rDNA will be positively identified in the coming years as being the cause of resistance development in a number of other clinical isolates. Experimental evidence pointing to this already exists in the case of the Mollicutes (*Mycoplasma* and *Ureaplasma*) (Palu et al., 1989, Stopler

and Branski, 1986), for which MLS antibiotics are also the therapeutic agents of choice.

Table 2:

Mutation	**Species**	**Phenotype**
C2611U	*E.coli*	$Ery^{R}Lin^{R}Sgb^{R}$
G2032A	*E.coli*	$Ery^{HS}Cln^{R}Cam^{R}$
G2032U	*E.coli*	$Ery^{HS}Cln^{S}Cam^{S}$
G2032C	*E.coli*	$Ery^{HS}Cln^{S}Cam^{S}$
G2057A	*E.coli*	Ery^{R}, Cam^{R}
A2058G	*E.coli*	Ery^{R}
A2058U	*E.coli*	Ery^{R}
A2058G	*M.intracellulare*	Cla^{R}
A2058C	*M.intracellular*	Cla^{R}
A2058U	*M.intracellular*	Cla^{R}
A2058G	*H.pylori*	Cla^{R}
A2059G	*H.pylori*	Cla^{R}
A2503C	*E.coli*	Cam^{R}

Table modified in accordance with the Weisblum, 1995, reference. Cam, chloramphenicol, Cla, clarithromycin, Cln, clindamycin, Ery, erythromycin, Lin, lincomycin, Sgb, streptogramin type B. HS, hypersensitive; R, resistant; S, sensitive.

The development of antibiotic resistances as a result of mutations in ribosomal nucleic acid sequences also has consequences for treating Helicobacter pylori infections. While the occurrence of spiral bacteria in the mucous membrane of the human stomach has been reported since the beginning of this century (Bizzozero, 1893), the fact that the organisms were pathogenic was only realized, and scientifically acknowledged, when Marshall and Warren (Warren and Marshall, 1983; Marshall et al., 1984) successfully isolated and cultured this bacterium from the mucous membrane of the stomach of a patient suffering from a gastric ulcer (ulcus ventriculi). As the first analyses

showed, the isolated microorganisms were Gram-negative, spiral bacteria which were extremely mobile and possessed the unusual ability to survive in strongly acid medium (up to approx. pH 1). Originally designated *Campylobacter pylori*, the organisms were finally grouped, on the basis of their biochemical and morphological properties, in the newly established genus *"Helicobacter"* (Goodwin et al., 1989).

The importance of *Helicobacter pylori* infection, and the consequence of this discovery, became clear within a few years. Epidemiological investigations carried out by Taylor and Blaser (1991) showed that *H. pylori* infection occurs worldwide and that approx. 50% of the population are infected with this bacterium, with the infection rate being higher in developing countries than in industrialized countries. Another observation is that the probability of a chronic infection with *H. pylori* increases drastically with increasing age. As a consequence, infections with *H. pylori* are among the most frequently occurring chronic bacterial infections in humans.

It is nowadays known that infection in humans inevitably leads to the triggering of a bacterial gastritis (type B gastritis). It is furthermore accepted as having been proved that the *H. pylori* infection also plays a causative role in the development of gastric and duodenal ulcers (ulcus ventriculi and ulcus duodeni) (Hentschel et al., 1991). According to a study carried out by Forman et al. (Forman et al., 1993), an *H.pylori* infection leads to a 6 to 12-fold increase in the risk of developing some forms of gastric carcinoma (adenocarcinoma). The more rarely occurring MALT (mucosa associated lymphoid tissue) lymphomas of the stomach, which are regarded as being the precursors of B cell tumors of the immune system, are also suspected of being a consequence of infection with *H. pylori*. Providing appropriate

patients with antibacterial treatment, involving the successful eradication (total elimination) of *H. pylori*, leads to both gastric ulcers and low-grade MALT lymphomas being healed (Sipponen and Hyvärinen, 1993; Isaacson and Spencer, 1993; Stolte and Eidt, 1993). On the basis of these data, *H. pylori* was classified by a World Health Organization (WHO) commission as being a Class 1 cancer-causing agent.

Before the existence of *H. pylori*, and its importance for ulcer diseases, was known, these diseases were treated with antacids or H_2 receptor antagonists. The latter are substances which inhibit the secretion of acid by the parietal cells of the stomach. The effect of these drugs is usually to cause the ulcers to heal; since, however, the drugs do not eliminate one of the causes of these ulcers, namely the infection with *H. pylori*, the ulceration recurs (recidivation) after a short period of time in most cases.

Another therapy which is frequently employed in association with ulcerations is that of bismuth treatment. Various bismuth salts (CBS, BSS) have a bactericidal effect on *H. pylori*. However, total eradication of the organism is only achieved in 8-32% of cases. While the treatment apparently leads to a transient suppression of the organism, the infection flares up again in most cases after the treatment has been discontinued. A more long-lasting therapy with high doses leads to an accumulation of the substance in the liver and kidneys and in the nervous system and has considerable neurological side effects (Malfertheiner, 1994).

Since it was realized that the gastroduodenal ulcer diseases were infectious diseases, an aim of the treatment has been that of eradicating the pathogens with antibiotics. However, monotherapy with various antibiotics (amoxycillin, tetracycline, nitrofuran,

furazolidine, and erythromycin, inter alia) turned out to be unsatisfactory since, with these drugs too, the organisms are only eradicated in 0-15% of the treatments. The previously recommended dual therapies using an acid blocker and an antibiotic also resulted in a high proportion of failures (Malfertheiner, 1994).

At present, the most successful treatment is achieved by the combination of an acid blocker (e.g. ompeprazole [sic]) and two antibiotics in the form of the "French" triple therapy (amoxicillin and clarithromycin) or, in the case of allergy to penicillin, the "Italian" triple therapy (clarithromycin and metronidazole), which triple therapies can lead to eradication rates of 80-95%. Because of its favorable properties, clarithromycin is increasingly becoming the agent of choice (Graham, 1995). A four-fold therapy (proton pump inhibitor, bismuth salt, tetracycline and metronidazole or clarithromycin) is recommended in the case of therapeutic failure due to the bacteria being resistant to clarithromycin or metronidazole, respectively. While this scheme promises success rates of about 95%, it nevertheless also suffers from substantial side effects.

The antibiotic resistance status of the bacteria is an important prerequisite for the success of the therapy. The failure rate of the treatment increases markedly as soon as there is resistance to only one of the two antibiotics employed (Buckley et al., 1997). The observation that the number of metronidazole-resistant and clarithromycin-resistant *H. pylori* isolates has been steadily increasing recently certainly gives cause for disquiet. While the reason for this is not known, it could lie in the increasing number of therapies for eradicating *H. pylori*, in particular the dual therapies using one antibiotic which have frequently been implemented in the recent past and in which resistances frequently appear. A resistance to macrolide

antibiotics such as clarithromycin or erythromycin is due to a point mutation in a particular region of the 23S rRNA. Such mutations apparently occur spontaneously and can readily be isolated by appropriate selection with an antibiotic. Rapid and reliable determination of the resistance status of *H. pylori* isolates prior to therapy is therefore of great importance for the future.

The simplest method of detecting an acute *Helicobacter pylori* infection with relative certainty is the so-called breath test (Desroches et al., 1997). This test measures the decomposition of orally administered, $^{13}C/^{14}C$-labeled urea into $^{13}CO_2$ or $^{14}CO_2$ and NH_4 by the bacterial urease. The $^{13}CO_2$ or $^{14}CO_2$ reaches the lungs via the blood circulation and is released in the lungs by way of the natural gas exchange and consequently becomes measurable in the respiratory air. The respiratory air is analyzed in a special appliance which is only possessed by special laboratories, so that the results are often obtained at a different site from that at which the samples were taken and there is consequently a delay in receiving them. Other noninvasive methods are based on PCR reactions which are carried out using stool or saliva samples (Schwarz et al., 1997). While these methods possess a very high degree of sensitivity, experience indicates that false-positive results occur very frequently in routine operation. Furthermore, these methods require a basic provision of apparatus and molecular biologicals which are not available in a general medical practice. This means that, in this case too, the samples have to be sent to special laboratories which means in turn that there is no possibility of obtaining the results rapidly. Indirect methods, which detect anti-Helicobacter antibodies in the serum or saliva, in turn suffer from the disadvantage that it is not possible to detect an acute infection beyond doubt.

As a rule, a gastroscopy is carried out when a positive breath test result has been obtained. A gastroscopy is often carried out in the case of a negative result as well, in particular when the symptoms do not subside after conventional therapy with proton blockers. As a rule, tissue samples are removed during a gastroscopy, in particular when it is possible to identify marked changes such as inflammations or ulcers. These tissue samples are examined histologically for benign or malignant tissue changes. Microbiological investigations, which are intended to assign the organism unambiguously and to determine its minimum inhibitory concentration (MIC) value, are carried out, in particular. These results make it possible to plan a specific therapy and consequently guarantee a high degree of success. In order to perform the microbiological investigations, it is necessary to culture the organism, a procedure which can take several days. After that, an MIC value determination is carried out, with this determination likewise being time-consuming and making it necessary to culture the organism. This investigation delays the beginning of the therapy substantially such that many doctors dispense with this investigation, which is to the disadvantage of the patient. The consequence of this is that doses of antibiotic which are too high, and which are accompanied by massive side effects, are employed in a therapy. On the other hand, the dose of antibiotic employed can be too low because no detection of antibiotic-resistant organisms was carried out. A rapid and reliable identification of the organism which is possibly causing the disease, and the immediate determination of its resistant status, are therefore of the utmost importance.

The invention relates to a novel process for determining, in microorganisms, in particular in bacteria, antibiotic reagents [sic] which are based on altered nucleic acid sequences, in particular in

ribosomal nucleic acids. When use is made of hybridization probes which are specific for a nucleic acid sequence which is associated with antibiotic resistances, it is surprisingly possible, in the case of microorganisms from a biological sample, to make rapid and reliable predictions about the presence and/or strength of an antibiotic resistance, or the minimum inhibitory concentration for an antibiotic, on the basis of the appearance or absence of a hybridization in an in-situ detection method. The present invention enables the attending physician to obtain results in an incomparably rapid and specific manner, thereby increasing the safety of the therapy for the patient and enabling the costs of the entire treatment to be reduced substantially.

Part of the subject-matter of the present invention is consequently a process for detecting antibiotic resistances in microorganisms, which process comprises the steps of:

a) preparing a sample which contains microorganisms,

b) bringing the sample into contact with at least one hybridization probe, which is specific for a nucleic acid sequence in microorganisms which is associated with antibiotic resistances, under conditions which permit the probe to hybridize selectively, and

c) analyzing the sample in situ by determining the appearance or absence of a hybridization.

Preferably, the process according to the invention for detecting antibiotic resistances is used in bacterial organisms. The nucleic acid sequence which is associated with antibiotic resistances is selected, in particular, from ribosomal nucleic acid sequences, particularly preferably from bacterial 23S ribosomal nucleic acid sequences. Particular preference is given to using nucleic acid sequences from the peptidyltransferase center on the 23S RNA, the sequence

of which (for E.coli) is depicted in Figure 1. Particularly preferably, the selected nucleic acid sequence encompasses a region corresponding to one or more of the nucleotides 2032, 2057, 2058, 2059, 2503 and 2611 on the E.coli 23S rRNA (numbering in accordance with Brosius et al., 1981).

However, in addition to this, the process according to the invention is also suitable for detecting antibiotic resistances in other microorganisms, e.g. in protozoa. Organisms which particularly come into consideration in this regard are those which can be controlled by the administration of macrolide antibiotics, e.g. Giardia lamblia, a protozoan pathogen which is found in the upper small intestine in man and which is the causative agent of lamblia dysentery (Jablonowski et al., 1997), or Pneumocystis carinii, a protozoan organism which can give rise to a pneumonia of frequently fatal outcome in immunodeficient patients, e.g. in HIV patients.

The process according to the invention has a number of advantages as compared with known methods for detecting antibiotic resistances. Thus, in contrast to classical biochemical detection methods, the process according to the invention can also be used for rapidly investigating slowly growing pathogens or pathogens which are difficult to culture, or cannot be cultured, in vitro. In addition to this, because the detection is in situ, it is possible to locate the organisms directly in affected areas of tissue. As compared with other molecular biological methods such as PCR, the process according to the invention is distinguished by the fact that time-consuming and labor-intensive DNA preparation methods are dispensed with and that the detection method is less sensitive to inhibitors which may possibly be present in the sample material. Furthermore, the nucleic acid which is detected can be associated with bacterial morphologies such as cocci, rods, etc., a feature which results in an improvement

in the reliability of the process. It is also possible to locate and quantify the pathogen in affected areas.

The process according to the invention is more specific and sensitive than other microscopic methods, e.g. staining methods (Gram, Grocott-Gomori, Giemsa). As compared with immunofluorescence methods, the smaller size of the probe is an advantage, which probe allows better penetration into the tissue, scarcely exhibits any nonspecific binding and is universally applicable and associated with lower costs.

The process according to the invention is suitable for all microorganisms, in particular pathogenic bacteria such as streptococci, Bordetella and Corynebacterium, and, in particular, for problematic microorganisms such as Helicobacter pylori, mycobacteria, Porphyromonas gingivalis, Borrelia burgdorferi, mycoplasmas, chlamydias and Tropheryma whippelii as well as representatives of the genera Bartonella, Legionella, Nocardia and Actinomyces, and also for other pathogenic organisms such as Pneumocystis carinii and Giardia lamblia.

A microorganism-containing sample is prepared in accordance with step (a) of the process according to the invention, preferably in the context of compiling results in human or veterinary medicine. While this sample can be any biological sample, it is preferably derived from human or animal tissues or body fluids, e.g. tissue sections, biopsies or blood samples. Surprisingly, the process according to the invention has such a high specificity and sensitivity that the sample can be investigated without the microorganisms being previously cultured or multiplied.

Particular preference is given to a presumptive medium being added to the sample prior to the investigation. This presumptive medium is a medium whose composition

is adapted to the microbial flora of the clinical sample, in particular to the disease-causing organisms which may possibly be present, and conserves these organisms in a viable state for a limited period of time, e.g. from several hours to several days, but to a large extent suppresses growth of the organisms. Such a presumptive medium essentially consists of a special nutrient solution which is present, where appropriate, in a semisolid organic matrix, e.g. an agar matrix. The nutrient solution contains a nitrogen source and essential components, such as trace elements, e.g. iron, zinc, manganese, vitamins, etc., which improve the stability of the isolated organisms. All the other constituents of this presumptive medium are preferably present in a buffered aqueous solution. The constituents of the nitrogen source are chemical or proteolytic digests of proteins of microbial, animal or vegetable origin, for example: peptones, tryptones or casitones or mixtures thereof, depending on the requirements of the selected microbial flora. In the case of anaerobic or microaerophilic organisms, reducing substances, such as reduced cysteine or thioglycolate, are admixed and/or oxygen-repelling additives are added to the medium.

Where appropriate, preparation of the sample can be additionally combined with an enrichment method, in particular when isolating organisms from liquid samples, e.g. blood or urine samples. The preferred enrichment method is based on binding the organisms to a solid matrix, in particular based on electrostatic interactions, e.g. to active charcoal or by way of defined ligands, such as polylysine, which are bound to a solid matrix. In accordance with the method, the blood flows, on withdrawal, into a sealed vessel, for example a syringe, which contains this matrix. After a brief incubation period, the blood is replaced by the abovementioned presumptive medium, with the matrix-

adsorbed organisms being conserved in a viable state in the sealed vessel.

Where appropriate, detection reagents, e.g. indicators which indicate the presence of pathogenic lead organisms within a short period of time, i.e. within minutes, can be added to the presumptive medium. This indicator can, for example, be a substrate mixture for a secreted lead enzyme (e.g. for the *H. pylori* urease) whose conversion leads to a chromogenic product and/or indicates the production of characteristic substances such as particular toxins or metabolites.

Prior to being examined for antibiotic resistances, the sample is also preferably fixed, e.g. with formaldehyde, paraformaldehyde or glutaraldehyde, and, if desired, permeabilized in order to enable the hybridization probes to penetrate more efficiently into the cells.

The hybridization probes may be nucleic acids such as DNA or RNA or else nucleic acid analogues or combinations of these. Preference is given to the hybridization probes being selected from nucleic acids such as DNA or nucleic acid analogues such as peptide nucleic acids (PNA).

The hybridization probes possess a hybridization region which is able to hybridize selectively with a target nucleic acid sequence in the microorganism. The length of this hybridization region preferably corresponds to from 15 to 20 nucleotide building blocks, in particular from 17 to 18 nucleotide building blocks. The hybridization probes employed in the process according to the invention are particularly suitable for detecting mutations, e.g. mutations of individual nucleotides or short nucleotide segments, which are selected from deletions, transversions, transitions and

modifications, e.g. methylations, of the corresponding wild type sequence.

The process according to the invention permits the use of a single hybridization probe. However, in many cases, it has proved advantageous to use a combination of several hybridization probes which are specific for different nucleic acid sequences associated with antibiotic resistances. The probe [sic] ClaR1 (SEQ ID NO. 1) and ClaR2 (SEQ ID NO. 2) are examples of hybridization probes according to the invention which can be used for detecting macrolide resistances at positions 2058 and 2059 of the 23S RNA. These probes can be used either singly or in combination.

In order to increase the sensitivity of the process according to the invention, the hybridization probes which are specific for mutations associated with antibiotic resistances can be used in combination with one or more hybridization probes which are specific for a nucleic acid sequence which is associated with the wild type of the microorganism (i.e. an antibiotic-sensitive strain). ClaWT (SEQ ID NO. 3), which can, for example, be used in combination with the previously mentioned probes ClaR1 and ClaR2, is an example of such a hybridization probe.

The ratio of resistant organisms to sensitive organisms in a sample can be determined by using wild type-specific probes and antibiotic resistances [sic] mutant-specific probes which each carry different labeling groups.

Furthermore, it is additionally possible to use, in the process according to the invention, a hybridization probe which is specific for a species or genus of microorganisms. Hybridization probes of this nature are known and are preferably directed specifically against ribosomal nucleic acid sequences, e.g. 23S RNA, 16S RNA

or ribosomal spacer sequences. Examples of these probes are the hybridization probes Hpy-1-16S-753 (SEQ ID NO. 4) and 120b (SEQ ID NO. 5), which are used for the species-specific detection of Helicobacter pylori.

Preference is given, in the process according to the invention, to using hybridization probes which carry a direct label, i.e. one or more labeling groups are linked directly to the probe, preferably by means of covalent bonding. On the other hand, it is also possible to use indirectly labeled or labelable probes, e.g. probes which carry a biotin group which is in turn detected by binding streptavadin, with the streptavadin being linked to a suitable labeling group. Alternatively, the hybridization probes can also contain sequence regions which do not hybridize with nucleic acid sequences in the microorganism and which can be employed for hybridizing with a further, complementary, (directly or indirectly) labeled probe.

Any labeling groups per se can be used for the process according to the invention provided they enable the in-situ detection to be sufficiently sensitive. Preference is given to dye groups, fluorescence groups and/or enzyme groups. Fluorescence labeling groups are particularly preferred.

If several types of hybridization probe (e.g. several mutation-specific probes or one or more mutation-specific probes in combination with wild type-specific probes and/or species-specific probes) are used in the process according to the invention, it may be advantageous to employ different labeling groups, i.e. labeling groups which can be detected alongside each other. Thus, it is possible, for example when additionally using species-specific probes which possess a label which is different from that of the mutation-specific probe, to obtain, when both the probes hybridize at the same time, a third and

different color which results from the two probe colors being mixed. If, for example, a green fluorophore, e.g. fluorescein, is chosen for the probes for determining the bacterial species, and a red fluorophore, e.g. rhodamine, is chosen for the probes for determining antibiotic resistance, the mixture of the two colors is then yellow. Using this probe combination, it is possible to determine the identity of the microorganism and the possible presence of an antibiotic resistance at one and the same time in one clinical sample.

In addition to this, the process according to the invention even makes it possible to draw additional conclusions with regard to the minimum inhibitory concentration (MIC) of antibiotic-resistant microorganisms. Thus, it has been found that particular point mutations, or particular combinations of point mutations, correlate directly with the MIC values determined by conventional means. MIC values can therefore be determined by using hybridization probes which detect particular point mutations or particular combinations of these point mutations. For example, it is possible to use hybridization probes which are specially prepared for low, medium and high MIC values. This makes it possible for the attending physician to use precise doses of the antibiotic for the therapy.

The hybridization probe according to the invention is specific for a nucleic acid sequence in microorganisms which is associated with antibiotic resistances. This means that hybridization conditions exist under which the hybridization probe hybridizes to a nucleic acid sequence which is associated with antibiotic resistances but not to a corresponding nucleic acid sequence from an antibiotic-sensitive wild type organism. Depending on the base sequence of the hybridization probe and the target sequence, the skilled person can readily ascertain hybridization conditions which are suitable for a particular test and

which provide adequate specificity in distinguishing between wild type and mutated sequences. The hybridization buffer employed is preferably a buffer which contains from 0.5 to 1.5 M salt, e.g. NaCl, a detergent, SDS and formamide. Washing preferably takes place in a formamide-free buffer. The probes are selected such that the hybridization temperature is preferably in the range from 45 to 55°C.

The sample is evaluated in situ, preferably by microscopic methods, e.g. using a fluorescence microscope.

Another part of the subject-matter of the invention is the use of an in-situ nucleic acid hybridization process for detecting antibiotic resistances in microorganisms, in particular in bacteria. The nucleic acid hybridization process is preferably carried out as previously described. The process is particularly suitable for detecting resistances to macrolide antibiotics. However, the process is also suitable for detecting antibiotic resistance mechanisms directed against lincosamide, aminoglycoside/aminocyclitol, tetracycline and chloramphenicol antibiotics and which are based on changes in ribosomal RNA sequences.

Particular preference is given to detecting resistances to macrolide antibiotics selected from the group consisting of clarithromycin, erythromycin, azithromycin and roxithromycin. In addition, particular preference is also given to detecting resistances to aminoglycoside antibiotics selected from the group consisting of streptomycin, neomycin, paromomycin, kanamycin, gentamicin, tobramycin, amikacin, netilmicin and sisomicin.

Yet another part of the subject-matter of the present invention is a reagent kit for typing microorganisms and/or detecting antibiotic resistances in

microorganisms. In a first embodiment, this reagent kit is based on detection by in-situ hybridization and comprises:

(a) means for preparing the sample, and

(b) at least one hybridization probe which is specific for a nucleic acid sequence in microorganisms which is associated with antibiotic resistances, and/or at least one hybridization probe which is specific for a species or genus of microorganism.

The means for preparing the sample preferably comprise a presumptive medium which can have a composition as previously indicated. Alternatively, or in addition, the means for preparing the sample can also comprise means for enriching microorganisms, e.g. an adsorption matrix for enriching organisms from a liquid sample, solutions or suspensions for fixing the sample, and hybridization buffer and/or washing buffer. The hybridization probes are preferably labeled as previously described.

According to a second embodiment according to the invention, the reagent kit for typing microorganisms and/or detecting antibiotic resistances in microorganisms comprises

(a) a presumptive medium for microorganisms, and

(b) means for typing and/or detecting antibiotic resistances.

According to this embodiment, the means for typing and/or for detecting antibiotic resistances are not restricted to using hybridization probes and other hybridization reagents. It is also possible to conceive of indicator substances which indicate the presence of microorganisms, e.g. urease detection reagents for Helicobacter pylori, hemolysin reagents for streptococci, and reagents for detecting toxins produced by Clostridium difficile, Corynebacterium diphtheriae and Bordetella pertussis. Preference is

given to the reagents for typing microorganism species or genera already being dissolved or suspended in the presumptive medium such that the kit contains a presumptive medium with combined indicator system. It is also conceivable for the presumptive medium with combined indicator system to be additionally combined with an enrichment method. In this case, the organisms are enriched from body fluids, for example blood, in a first step and treated, in a second step, with the indicator-containing presumptive medium. In addition, the kit can contain, where appropriate in separate form, means for detecting antibiotic resistances, e.g. the previously described hybridization probes, and suitable buffers.

In addition to this, the invention relates to the use, for the species-specific detection of *Helicobacter pylori*, of an oligonucleotide having a nucleotide sequence from a region of the V domain of the 16S rRNA. It was ascertained, surprisingly, that, when used as amplification primers or hybridization probes, such oligonucleotides, i.e. nucleic acids or nucleic acid analogues having a length of preferably from 10 to 30 nucleotide building blocks, exhibit a significantly higher specificity with regard to recognizing different H.pylori isolates than do other sequences and, in addition to this, enable H.pylori and other related species to be differentiated reliably. Preference is given to using an oligonucleotide which contains the sequence depicted in SEQ ID NO. 4 or at least a part region thereof which is 10 nucleotides in length. It is furthermore preferred that the oligonucleotide carries a labeling group.

Yet another part of the subject-matter of the invention is the use of an oligonucleotide from a bacterial 23S rRNA, in particular from a region which contains the peptidyltransferase center, for detecting antibiotic resistances. Preference is given to using

oligonucleotides which are specific for a mutation of the wild type sequence which is associated with antibiotic resistances and encompass a region corresponding to one or more of the nucleotides 2032, 2057, 2058, 2059, 2503 and 2611 on the E.coli 23S rRNA. Particular preference is given to using oligonucleotides having the sequence depicted in SEQ ID NO. 1 or SEQ ID NO. 2. Where appropriate, these antibiotic resistance-specific oligonucleotides can be used together with a wild type-specific oligonucleotide which originates from the same region as the resistance-specific probe. The wild type-specific oligonucleotide preferably has the sequence depicted in SEQ ID NO. 3.

Consequently, the present invention also relates to oligonucleotides, i.e. nucleic acids and/or nucleic acid analogues, which contain the sequence depicted in SEQ ID NO. 1, 2, 3 or 4 or at least a part region thereof which is 10 nucleotides in length. The invention also relates to compositions which contain two or more of said oligonucleotides. One or more of the oligonucleotides preferably carry a labeling group.

The invention is explained further by means of the following figures and examples.

Fig. 1 shows a diagram of the peptidyltransferase center in bacterial 23S rRNA. The diagram is a linear representation in which the positions at which resistance mutations have already been found are indicated by underlining. Filled circles represent the footprints of erythromycin. The upper numbers relate to the helix numbering according to Brimacombe and the lower numbers relate to the nucleotide position in E.coli according to Brosius.

SEQ ID NO. 1-5 show the nucleotide sequences of the probes ClaR1 (1), ClaR2 (2), ClaWT (3), Hpyl-165-753 [sic] (4) and 120b (5).

Examples

Example 1

Detection of *H. pylori* using species-specific probes.

An alignment comprising 108 almost complete 16S rRNA sequences from organisms from the ∈ group of proteobacteria (including 50 *Helicobacter* sequences, in turn including 10 *H. pylori* sequences) was used to develop species-specific *H. pylori* probes. The probe Hpyl-16S-753 is directed against a region of the V domain of the 16S rRNA (Neefs et al., 1993), which only occurs in *H. pylori* and is not found in other bacterial species (Table 3). A further probe, 120b, is directed against another *H. pylori*-specific region of *H. pylori* 16S rRNA (Table 3). The two probes were provided either with the fluorescence dye fluorescein (emits green fluorescence) or the fluorescence dye Cy3 (emits red fluorescence) by way of an amino linker at the 5′ end.

Table 3: Nucleotide sequences of different probes

Name	Sequence (5′-3′)	Target region*	Specificity
Hpyl-16S-753	GCTTTCGCGCAATCA GCG	753-770 (16S)	H. pylori
120 b	AGGCACATGATCTAT GCG	120-137 (16S)	H. pylori
ClaR1	CGGGGTCTTCCCGTC TT	23S	A2059G (Cla^R)
ClaR2	CGGGGTCTCTCCGTC TT	23S	A2058G (Cla^R)
ClaWT	CGGGGTCTTTCCGTC TT	23S	Wild type (Cla^S)

* According to Brosius et al., 1981

Different reference cells are fixed with 3% buffered paraformaldehyde solution, as described in Amann et al., and immobilized on microscope slides by means of air drying (Amann et al., 1990).

5 ng of the probe are incubated with these microscope slides at 46°C for 90 min, in a hybridization buffer (0.9 M NaCl, 0.02 M tris/HCl, pH 8.0, 0.01% SDS, 20% formamide). The slides are then washed for 15' at 48°C (0.225 M NaCl, 0.02 M tris/HCl, pH 8.0, 0.01% SDS). Excess washing buffer is removed from the microscope slides with PBS and the slides are embedded in Citifluor AF1 (Citifluor Ltd., London, UK) in order to decrease color-fading effects. The hybridization is analyzed using an epifluorescence microscope (standard filters for red and green fluorescence). Under the given conditions, all the *H. pylori* strains which have so far been tested (16/16) hybridized with the probe Hpyl-16S-753 whereas cells from other *Helicobacter* species and other reference strains (6/6) did not bind to the probe (Table 4). These other *Helicobacter* species and further reference strains were the species which are most closely related to *Helicobacter pylori*, i.e. *H. mustelae, H. felis, H.fennelliae, C. coli, C. jejuni* and *W. succinogenes* (Table 4). Probe 120b reacted with 11 out of 16 strains and thereby displayed a somewhat lower specificity with regard to recognizing different *H. pylori* isolates (Table 4).

Table 4: Testing the specificity of two *H. pylori* probes

Strain	Probe 120b2 (red)	Hpy1-16S-753 (red)
H. pylori P1	++	++
H. pylori P79	++	++
H. pylori P79B6.1	n.d.	n.d.
H. pylori P2	++	++
H. pylori P8	++	++
H. pylori P12	-	++
H. pylori P80	n.d.	n.d.
H. pylori P80B6.1	-	++
H. pylori P21	++	++
H. pylori P27	++	++
H. pylori P29	++	++
H. pylori P31	++	++
H. pylori P49	++	++
H. pylori P76	++	++
H. pylori P66	++	++
H. pylori P92^r	-	++
H. pylori P106	-	++
Cl^r	-	++
Helicobacter musteleae NCTC12032	n.d.	-
Helicobacter felis ATCC49179	n.d.	-
Helicobacter fennelliae	n.d.	-
Campylobacter coli	n.d.	-
Campylobacter jejuni	n.d.	-
Wolinella succinogenes	n.d.	-

++, good hybridization; -, no hybridization

Example 2

Using rRNA-directed, fluorescence-labeled oligonucleotide probes for the detection of clarithromycin-resistant *H. pylori*.

Two different mutations in the 23S rRNA have by now been found which are able to mediate resistance to the macrolide clarithromycin (Versalovic et al., 1997). Resistances can be caused by an A = > G transitions [sic] either at E. coli position 2058 or at 2059. Two probes were constructed (Table 3) in order to be able

to detect both mutations at the same time. Probe ClaR2 is complementary to the rRNA which is altered at position 2058 while probe ClaR1 is complementary to the rRNA which is mutated at position 2059. Probe ClaWT, which is complementary to the wild-type sequence in this region, was constructed as well. Since the resistant mutant and wild type only differ by one base, a competitive hybridization assay was chosen. This assay used the two Cy3-labeled probes ClaR1 and ClaR2 together with an equimolar quantity of a FLUOS-labeled ClaWT probe. The probes were prepared in analogy with Example 1.

The specificity tests carried out on 23 resistant and 10 sensitive *H. pylori* strains showed that a hybridization buffer of the following composition: (0.9 M NaCl; 0.02 M Tris/HCl pH 8.0; 0.01% SDS; 20% formamide) guarantees 100% specificity when this competitive assay is used. The results obtained from this hybridization are presented in Table 5. Otherwise, the in-situ hybridization was carried out under the same conditions as specified in Example 1.

Under the fluorescence microscope, the *Helicobacter* strains which carry the wild-type sequence then fluoresce green and can be readily distinguished from the red-fluorescent, resistant Helicobacter strains which possess a 23S rRNA mutation. The H. pylori specificity can additionally be detected with the Hpyl-16S-753 probe. When combinations of Hpyl-16S-753 (green) and ClaR1 or ClaR2 (red) are used, clarithromycin-resistant H. pylori mutants then appear yellow due to the colours being mixed whereas clarithromycin-sensitive H. pylori only react with Hpyl-16S-753 and therefore fluoresce green.

Table 5: Testing the specificity of the clarithromycin-resistance probes ClarR1 and Clar2 and of the wild-type sequence probe ClaWT

Strain	MHC (E test; mg/l)	ClaWT probe (green)	ClaR1 probe (red)	ClaR2 probe (red)	Notes
F569					n.d.
MC098	>256				n.d.
Clr337	n.d.		++		
Clr	>256		++		
WM	n.d.		++		
BJ550	>256		++		
MC081	>256		++		
MC141	>256		++		
F609	n.d.		+		
EP14	n.d.	++	++	++	Mixture
BM512	32	++	++	++	Mixture
MC132	12	++	++	++	Mixture
EP14	n.d.			++	
F675	n.d.			++	
Ka25	n.d.			++	
LK11	n.d.			++	
Si203	n.d.			++	
FD591	16/32			++	
HH531	48			++	
KJ472	16			++	
MC028	24			++	
F569	n.d.			+	
JW22	n.d.	++			
LG400	>256	++			
MC099	>256	++			
96	n.d.				n.d.
JW22	n.d.				n.d.
LK5	n.d.				n.d.
C160	n.d.				n.d.
FD591	16/32				n.d.

Probes: ClaWT, probe which recognizes the wild-type sequence, labeled red; ClaR1 recognizes the mutation

A2059G (Cla^R) and ClaR2 recognizes the sequence A2058G (Cla^R) (see Table 3); n.d., not determined. ++, good hybridization; +, weak hybridization,

The table shows a correlation between the position of the mutation and the magnitude of the MHC for resistance to clarithromycin. Mutations in position A2059G, as detected with the ClaR1 probe, have significantly higher MIC values than do mutations in A2058G. This correlation has already been described by Versalovic et al., (1997).

Example 3

Detecting *H. pylori* in a mouse stomach tissue section using specific rRNA-directed probes

6-8-week-old C57Bl6 mice are infected perorally with 10^8 colony-forming units of the *H. pylori* strain P76. The mouse is killed 4 days after the inoculation and the stomach is removed under sterile conditions. The scalpel is used to chop the stomach into small pieces which fit into an Eppendorf tube; the pieces are then fixed in a 3% solution of paraformaldehyde for 12 h. The tissue pieces are then washed 2 × 1 h in PBS and frozen at -70°C in a frozen section medium. A cryomicrotome is used to prepare 5-10 µm-thick frozen sections of the stomach, with the sections then being immobilized on polylysine-coated slides. The hybridization is carried out as explained in Example 1. Individual *H. pylori* cells can be clearly detected on the inner sides of the crypts in the stomach sample.

Example 4

Comparing the 23S rRNA sequences of different medically important bacteria within the clarithromycin resistance region.

A computer program was used to compare the 23S rDNA [sic] from a further 40 Gram-positive and Gram-negative bacteria with the corresponding region of *H. pylori* and *E. coli* (Table 6). As the results show, this region of the ribosomal RNA is strongly conserved in all the bacteria used for the comparison. However, ClaR1 and ClaR2 probes are seen to be specific. The 40 sequences from the database were presumably derived from clarithromycin-sensitive bacteria. If clarithromycin resistance in these bacteria is also associated with a mutation in position 2058/2059 in the 23S rDNA [sic], the method described in Example 2 can then be applied directly to these bacteria. It is only necessary, for the purpose of identifying the species, to combine the resistance probe with a species-specific probe. The described method then has an enormously broad potential for detecting resistances in a large reservoir of medically important bacteria.

Table 6:

Comparison of the 23S rRNA sequences of various bacterial species within the clarithromycin resistance region

Probe sequence			5´-CGGGGTCTTTCCGTCTT-3
rRNA sequence	mis	E.coli	5´-AAGACGGAAAGACCCCG-3´
Helicobacter pylori claWT	0	2051	ACCCGCGGC-==========-UGGACCUUU
Helicobacter pylori claR1	1	2051	ACCCGCGGC-=======G=========-UGGACCUUU
Helicobacter pylori claR2	1	2051	ACCCGCGGC-========G========-UGGACCUUU
Campylobacter jejuni	0	2051	ACCCGCGGC-==========-UGGACCUUU
Campylobacter coli	0	2051	ACCCGCGGC-==========-UGGACCUUU
Wolinella succinogenes	0	2051	ACCCGCGGC-==========-UGGACCUUU
Nannocystis exedens	0	2051	ACCCGCGGC-==========-UGAACCUUU
Escherichia coli	0	2051	ACCCGCGGC-==========-UGAACCUUU
Salmonella typhi	0	2051	ACCCGCGGC-==========-UGAACCUUU
Enterobacter cloacae	0	2051	ACCCGCGGC-==========-UGAACCUUU
Citrobacter freundii	0	2051	ACCCGCGGC-==========-UGAACCUUU
Klebsiella pneumoniae	0	2051	ACCCGCGGC-==========-UGAACCUUU
Yersinia pestis	0	2051	ACCCGCGGC-==========-UGAACCUUU
Plesiomonas shigelloides	0	2051	ACCCGCGGC-==========-UGAACCUUU
Haemophilus influenzae	1	2051	ACCCGCGGC-U=========-UGAACCUUU
Vibrio vulnificus	1	2051	ACCCGCGGC-U=========-UGAACCUUU
Aeromonas hydrophila	1	2051	ACCCGCGGC-U=========-UGAACCUUU
Pseudomonas aeruginosa	1	2051	AUCCGCGGC-U=========-UGAACCUUU
Acinetobacter calcoaceticus	1	2051	ACCCGCGGC-U=========-UGAACCUUU
Neisseria meningitidis	1	2051	ACCCGCUGC-U=========-UGAACCUUU
Bordetella pertussis	2	2051	ACCCGCGGC-U==========A-UGAACCUUU
Bartonella bacilliformis	1	2051	UCCUGCGGU-U=========-UGCACCUUU
Rickettsia rickettsii	1	2051	UCCCGCGGU-C=========-UGAACCUUU
Borrelia burgdorferi	1	2051	ACUUGUGGU-U=========-UGAACCUUU
Leptospirillum ferrugineum	2	2051	CCCCGCGGC-U==========U=-UGCACCUUU
Listeria monocytogenes	1	2051	ACCCGCGAC-=G=========-UGGAGCUUU
Staphylococcus aureus	1	2051	ACCCGCGAC-=G=========-UGGAGCUUU
Bacillus anthracis	1	2051	ACCCGCGAC-=G==========N=-UGGAGCUUU
Mycoplasma hyopneumoniae	1	2051	ACCCGCAUC-======A=========-UGGAGCUUU
Mycoplasma pneumoniae	2	2051	AGGCGCAAC-GG=========-UGAAGCUUU
Streptococcus parauberis	2	2051	ACCCGCGAC-=G==========A-UGGAGCUUU
Lactococcus lactis	2	2051	ACCCGCGAC-=G==========A-UGGAGCUUU
Enterococcus faecalis	2	2051	ACCCGCGAC-=G==========A-UGGAGCUUU
Clostridium botulinum	2	2051	ACCCGCGAU-UG=========-UAGAGCUUU
Streptomyces griseus	1	2051	UCGCGCAGC-=G==========-GGACCUUUA
Micrococcus luteus	1	2051	ACGCGCAGA-=G==========-UGACCUUUA
Corynebacterium glutamicum	1	2051	ACGCGCGGC-=G==========-GGACCUUCA
Gardnerella vaginalis	1	2051	AAGCGCAGA-=G==========-GGACCUUUA
Mycobacterium leprae	2	2051	ACGUGCGGC-=G====A=========-GGACCUUCA
Bifidobacterium bifidum	2	2051	AAGCGCAGA-=G====A=========-GGACCUUUA
Chlamydia trachomatis	2	2051	ACCCGCGAA-=G====A=========-UGAACCUUU
Chlamydia pneumoniae	2	2051	CCCCGCAAA-=G====A=========-UGAACCUUU
Bacteroides fragilis	3	2051	ACCCGCGAU-GG====A=========-UGAACCUUU

mis, number of mismatches with the rRNA sequence in this region. E. coli, the starting position of the probe sequence in the 23S rRNA of the different species relative to the position in E. coli (Brosius et al., 1981), N, corresponds to A, C, G or T. = identical to the rRNA sequence.

Example 5

Developing a presumptive medium for bacterial gastritis combined with urease detection especially for *Helicobacter pylori*

The particular aim is to develop a presumptive medium which should make it possible to conserve viable *Helicobacter pylori* bacteria from stomach biopsies, ideally over a period of 5 days but at least over 48 hours. In addition, the accompanying flora, if present, should be preserved in order to ensure that the microbiological evaluation is complete. The growth of the organisms must be restricted in order to keep the fractional composition of the organisms in the biopsy as unaltered as possible. The presumptive medium essentially consists of a buffered nutrient solution which is preferably present in a semisolid organic matrix, such as (0.2-1.5%) agar or (at least 15%) gelatin. The nutrient solution contains a nitrogen source and further essential components which improve the stability of Helicobacter. The nitrogen source takes up from 0.5 to 5% of the presumptive medium. It consists of chemical or proteolytic digests of proteins of microbial, animal or plant origin, such as peptones, tryptones or casitones or mixtures of these compounds. Particular preference is given to media such as Schivo-Medium® or brain-heart infusion (BHI)-based media. Other preferred constituents are yeast extract (e.g. 0.01%), serum proteins, such as horse serum or fetal calf serum or bovine serum albumin, and defined organic substances, such as 2,6-dimethyl-beta-cyclodextrin and cholesterol. The serum proteins should take up 1-10% of

the presumptive medium, with the organic substances taking up 0.01-0.2%. In addition, reduced cysteine or thioglycolate are admixed with the presumptive medium and/or oxygen-repelling additives are added to it. All the components are present in a buffered, aqueous solution whose preferred pH is between 5.5 and 6.5. The biopsy sample is introduced into the lower third of the semisolid matrix thereby preventing atmospheric oxygen from diffusing in, an arrangement which improves the survival conditions for the organism.

Combining the presumptive medium with a urease detection test is novel. It offers the physician the advantage that he is given a rapid indication of a possible *Helicobacter* infection and can directly initiate further measures. To this end, (0.5-5%) urea and (0.001-0.01%) phenol red, or other pH indicators, such a bromcresol purple, are also added to the presumptive medium. The transformation of the urea by urease leads, inter alia, to the production of ammonia, which makes the pH basic and, for example, changes the pH indicator phenol red from yellowish into red to violet. The speed of the color change, the intensity of the color, and the coloration, correlate directly with the quantity of urease-producing bacteria. The constituents of the combined presumptive medium are adjusted such that high sensitivity is achieved (500-2000 organisms/presumptive medium) and autolysis of the organisms is suppressed. Alternatively, a synthetic urease substrate, whose transformation brings about a color change, can be admixed with the presumptive medium.

Comparative investigations provide evidence that other, routinely employed urease detection methods lead to rapid lysis of the organisms. This is of the greatest importance since more recent investigations show that other urease-producing organisms, such as *Proteus mirabilis, Klebsiella oxytoca* and *Pseudomonas*

aeruginosa, are frequently to be found in the biopsy samples. In contrast to *Helicobacter*, these organisms do not secrete any urease; instead the enzyme is present inside the cell. In our own investigations, we have established that the bacteria are lysed in current urease detection methods such as the CLO test, with the intracellular urease being released and immediately reacting with the substrate which is now available. In a provisional investigation carried out on 32 patients suffering from acute gastritis, it was only possible, when conventional methods were used, to isolate a *Helicobacter* from 34% of the biopsies which were examined microbiologically. In this connection, the possibility cannot be ruled out that the *Helicobacter*-free biopsies were colonized with strains which are very difficult to culture. However, it is significant that a major part (> 50%) of the *Helicobacter*-free biopsies contained other urease-producing organisms which were detected by the currently employed methods for detecting urease. The combined transportation and indicator medium delays or prevents lysis of these bacteria and consequently guarantees that the detection of urease is *Helicobacter*-specific.

Table 7:

Urease-producing organism[1]	Change in color of the presumptive medium[2]	
	5 hours	24 hours
Helicobacter pylori	++ (< 10 minutes)	+++
Proteus mirabilis	+/- (> 3 hours)	++
Klebsiella oxytoca	-	-
Pseudomonas aeruginosa	-	-

[1] One complete bacterial colony was used for each test.

2 The presumptive medium was incubated at room temperature (21-25°C) and evaluated visually.

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Claims

1. Process for detecting antibiotic resistances in microorganisms, comprising the steps of:
   a) preparing a sample containing microorganisms,
   b) bringing the sample into contact with at least one hybridization probe, which is specific for a nucleic acid sequence in microorganisms which is associated with antibiotic resistances, under conditions which permit the probe to hybridize selectively, and
   c) analyzing the sample in situ by determining the appearance or absence of a hybridization.

2. Process according to Claim 1,
characterized in that
the microorganisms are selected from bacterial organisms and protozoa.

3. Process according to Claim 1 or 2,
characterized in that
the nucleic acid sequence which is associated with antibiotic resistances is selected from ribosomal nucleic acid sequences.

4. Process according to Claim 3,
characterized in that
the nucleic acid sequence is selected from bacterial 23 S ribosomal nucleic acid sequences.

5. Process according to Claim 4,
characterized in that
the nucleic acid sequence encompasses a region corresponding to one or more of the nucleotides 2032, 2057, 2058, 2059, 2503 and 2611 on the E.coli 23S rRNA.

6. Process according to one of the preceding claims,
characterized in that

slowly growing pathogens and/or pathogens which are difficult to culture, or cannot be cultured, in vitro, are tested.

7. Process according to Claim 6, characterized in that the microorganisms are selected from the group consisting of Helicobacter pylori, mycobacteria, Bacteroides gingivalis, Borrelia burgdorferi, mycoplasmas, chlamydias, Tropheryma whippelii, bartonellas, legionellas, norcardias [sic] and actinomycetes.

8. Process according to one of the preceding claims, characterized in that use is made of a sample which is derived from human or animal tissues or body fluids.

9. Process according to one of the preceding claims, characterized in that the sample is investigated without the microorganisms being previously cultured.

10. Process according to one of the preceding claims, characterized in that the sample is subjected to a procedure for enriching microorganisms.

11. Process according to one of the preceding claims, characterized in that a presumptive medium is added to the sample prior to the investigation.

12. Process according to Claim 11, characterized in that the presumptive medium contains an indicator substance for typing microorganisms.

13. Process according to one of the preceding claims,

characterized in that
the sample is fixed, and, where appropriate, permeabilized, prior to the investigation.

14. Process according to one of the preceding claims, characterized in that
the hybridization probe is selected from nucleic acids such as DNA or nucleic acid analogues such as PNA.

15. Process according to one of the preceding claims, characterized in that
the hybridization probe possesses a hybridization region having a length corresponding to from 15 to 20 nucleotide building blocks, in particular from 17 to 18 nucleotide building blocks.

16. Process according to one of the preceding claims, characterized in that
use is made of a hybridization probe which is specific for mutations selected from deletions, transversions, transitions and modifications of the corresponding wild type sequence.

17. Process according to one of the preceding claims, characterized in that
use is made of a combination of several hybridization probes which are specific for different nucleic acid sequences associated with antibiotic resistances.

18. Process according to either Claim 16 or 17, characterized in that
use is made of the hybridization probes ClaR1 (SEQ ID NO. 1) and/or ClaR2 (SEQ ID NO. 2).

19. Process according to one of the preceding claims, characterized in that

use is additionally made of at least one hybridization probe which is specific for a nucleic acid sequence which is associated with a wild type of the microorganism.

20. Process according to Claim 19, characterized in that use is made of the hybridization probe ClaWT (SEQ ID NO. 3).

21. Process according to one of the preceding claims, characterized in that use is additionally made of at least one hybridization probe which is specific for a species or a genus of microorganism.

22. Process according to Claim 21, characterized in that use is made of the hybridization probes Hpy1-165-753 [sic] (SEQ ID NO. 4) and/or 120b (SEQ ID NO. 5) to detect Helicobacter pylori.

23. Process according to one of the preceding claims, characterized in that use is made of hybridization probes which carry a direct label.

24. Process according to one of the preceding claims, characterized in that use is made of hybridization probes which are labeled, or can be labeled, with dye groups, fluorescence groups and/or enzyme groups.

25. Process according to one of the preceding claims, characterized in that use is made of several hybridization probes which are labeled, or can be labeled, differently.

26. Process according to one of the preceding claims,

characterized in that
the sample is analyzed by microscopic methods.

27. Process according to one of the preceding claims, characterized in that
the analysis comprises quantitatively determining antibiotic resistances.

28. Use of an in-situ nucleic acid hybridization process for detecting antibiotic resistances in microorganisms.

29. Use according to Claim 28 for detecting antibiotic resistances in bacteria and protozoa.

30. Use according to Claim 28 or 29 for detecting resistances to macrolide, lincosamide, aminoglycoside, aminocyclitol, tetracycline and chloramphenicol antibiotics.

31. Use according to Claim 30 for detecting resistances to macrolide antibiotics selected from the group consisting of clarithromycin, erythromycin, azithromycin and roxithromycin.

32. Use according to Claim 28 or 29 for detecting resistances to aminoglycoside antibiotics selected from the group consisting of streptomycin, neomycin, paromomycin, kanamycin, gentamicin, tobramycin, amikacin, netilmicin and sisomicin.

33. Reagent kit for typing microorganisms and/or antibiotic resistances in microorganisms by in-situ hybridization, comprising
(a) means for preparing the sample, and
(b) at least one hybridization probe which is specific for a nucleic acid sequence in microorganisms which is associated with antibiotic resistances, and/or at least one

hybridization probe which is specific for a species or genus of microorganisms.

34. Reagent kit according to Claim 33, characterized in that the means for preparing the sample comprise a presumptive medium and, where appropriate, means for enriching microorganisms.

35. Reagent kit according to Claim 34, characterized in that the presumptive medium contains a nutrient solution containing a nitrogen source and other essential components and also, where appropriate, reducing substances and/or oxygen-repelling additives.

36. Reagent kit for typing microorganisms and/or [lacuna] antibiotic resistances in microorganisms, comprising

(a) a presumptive medium for microorganisms, and

(b) means for typing microorganisms and/or for detecting antibiotic resistances.

37. Reagent kit according to Claim 36, characterized in that the presumptive medium contains a nutrient solution containing a nitrogen source and other essential components and also, where appropriate, reducing substances and/or oxygen-repelling additives.

38. Reagent kit according to Claim 36 or 37, characterized in that the means for typing microorganisms comprise indicator substances which are dissolved and/or suspended in the presumptive medium.

39. Reagent kit according to Claim 38,

characterized in that
it contains a urease indicator for detecting Helicobacter pylori.

40. Use of a reagent kit according to one of Claims 33 to 39 in a process according to one of Claims 1 to 27.

41. Use of an oligonucleotide from a region of the V domain of the 16S rRNA for the species-specific detection of H. pylori.

42. Use according to Claim 41,
characterized in that
the oligonucleotide contains the sequence depicted in SEQ ID NO. 4, or at least a part region thereof which is 10 nucleotides in length.

43. Oligonucleotide,
characterized in that
it contains the sequence depicted in SEQ ID NO. 4, or at least a part region thereof which is 10 nucleotides in length.

44. Oligonucleotide according to Claim 43,
characterized in that
it carries a labeling group.

45. Use of an oligonucleotide from a bacterial 23S rRNA for detecting antibiotic resistances.

46. Use according to Claim 45,
characterized in that
the oligonucleotide contains the sequence depicted in SEQ ID NO. 1 or SEQ ID NO. 2.

47. Use according to Claim 45 or 46 together with a wild type-specific oligonucleotide, in particular an [sic]

47. Use according to Claim 45 or 46 together with a wild type-specific oligonucleotide, in particular an oligonucleotide which contains the sequence depicted in SEQ ID NO. 3.

48. Oligonucleotide,
characterized in that
it contains the sequence depicted in SEQ ID NO. 1, 2 or 3, or at least a part region thereof which is 10 nucleotides in length.

49. Oligonucleotide according to Claim 48,
characterized in that
it carries a labeling group.

Abstract

The invention relates to a process for detecting antibiotic resistances in microorganisms, in particular in bacteria, and to reagent kits which are suitable for implementing the process.

sh/ANM/18608PDE 22.05.1998

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Creatogen Biosciences GmbH
(B) STREET: Ulmerstrasse 160a
(C) CITY: Augsburg
(E) COUNTRY: Germany
(F) POSTAL CODE: 86156

(ii) TITLE OF INVENTION: Detection of antibiotic resistances in microorganisms

(iii) NUMBER OF SEQUENCES: 5

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: Nucleotide
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CGGGGTCTTC CCGTCTT

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: Nucleotide
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CGGGGTCTCT CCGTCTT

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 base pairs
(B) TYPE: Nucleotide
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

CGGGGTCTTT CCGTCTT

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: Nucleotide
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GCTTTCGCGC AATCAGCG

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs
(B) TYPE: Nucleotide

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

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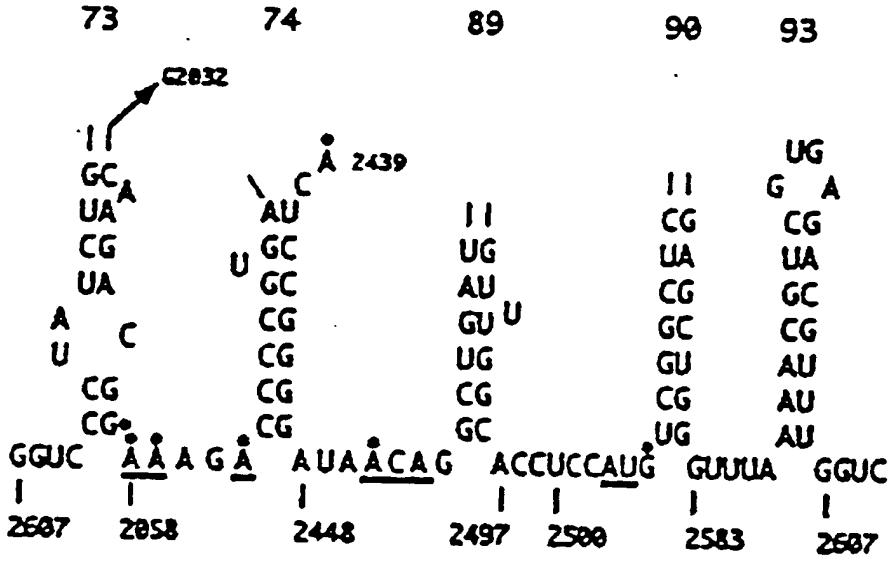
AGGCACATGA TCTATGCG

Fig. 1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